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Mechanisms of selective toxicity of metronidazole and other nitroimidazole drugs

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SUMMARY The selectively toxic effect of nitroimidazole drugs towards anaerobic bacteria and protozoa depends on a number of factors. The killing action of such drugs as metronidazole requires the reduction of the nitro group, a process which influences the rate of entry of the drug into the susceptible cell and which is determined by mechanisms involving ferredoxin-linked (or the equivalent) reactions in the cell. The reduced agent subsequently causes strand breakage of DNA, the extent of which depends on the A + T content of the DNA. Other effects of such drugs may include the possible inhibition of DNA repair mechanisms which exacerbate DNA damage. Inhibition of activity of nitroimidazoles may be caused by aminothiol radical scavengers and radioprotectors normally present in the cell or by the presence of other organisms in the environment (that is, the vagina) capable of inactivating the drugs.

Introduction

In 1978, an editorial in this journal[1] gave a short review of the types, availability, and relative activity of nitroimidazoles used primarily against *Trichomonas vaginalis* infections. It is now timely to review the mechanism of action of such drugs in an attempt to explain their selective action and their unique contribution to the chemotherapy of anaerobic organisms since their introduction in 1959.

The first of these drugs, metronidazole, has been studied more widely than any other and consequently will be used as the type example. Metronidazole (1-β-hydroxyethyl-2-methyl-5-nitroimidazole) is effective against a variety of protozoal diseases including trichomoniasis,[2] giardiasis,[3] amoebiasis,[4] and balantidiasis,[5] as well as being a valuable agent for the treatment of bacterial disease caused by anaerobes—for example, Vincent's disease[6] and post-operative infections.[7 8] More recently, much interest has been shown in its possible application as a specific radiosensitiser of hypoxic tumours together with the 2-nitroimidazole, misonidazole.[9-13]

Other nitroimidazole drugs, such as tinidazole, sulnidazole, secnidazole, and ornidazole, have broadly similar spectra of activity but none has sufficient advantages to supplant metronidazole from its role as the drug of choice for many bacterial and protozoal diseases. The structure of the drugs is shown in the figure. The spectrum of activity of nitroimidazoles is unique in that unlike any other antimicrobial drug they encompass Gram-positive and Gram-negative bacteria, protozoa, and even a few nematode worms, all of which are either anaerobic or micro-aerophilic. Thus the selective toxicity of these drugs rests on their ability to exert a cytotoxic effect specific to anaerobes.

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Mode of action

The first studies on the mode of action of metronidazole indicated that it inhibited the output of H_2 gas from *T vaginalis* before that of CO_2, which parallels cell death, indicating that H_2 inhibition is a primary effect.[14] Further studies showed that H_2 output was by a phosphoroclastic mechanism of the clostridial type, in which pyruvate and phosphate are converted to acetylphosphate, CO_2, and H_2, and that nitroimidazoles act by accepting electrons from an electron transport protein which is normally involved in H_2 gas evolution.[14 15] Since other drugs, for example, dimetridazole and tinidazole, have the same effect this inhibition appears to be a general property of 5-nitroimidazoles. However, the impaired function of the phosphoroclastic mechanism is not

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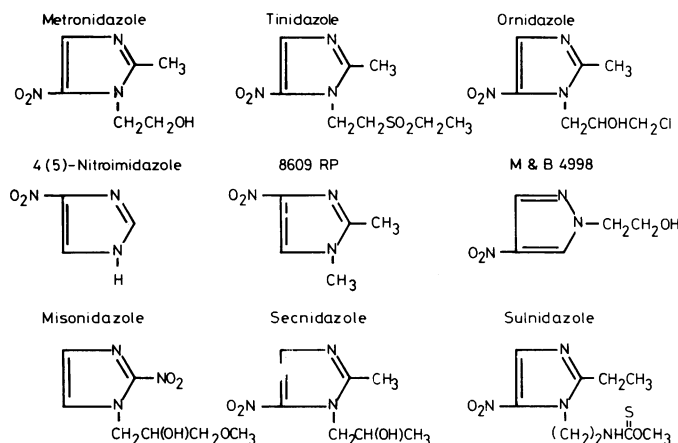

FIGURE *Structures of nitroimidazole drugs. Metronidazole, tinidazole, ornidazole, secnidazole, and sulnidazole are 5-nitroimidazoles, and carnidazole is the 2-methyl congener of sulnidazole. Misonidazole is a 2-nitroimidazole, 8609 RP, a 4-nitroimidazole, and M & B 4998, a 4-nitropyrazole.*

responsible for death since the cell recovers once all the drug is reduced.[16]

Examination of the reduction process showed that the key step lies in the potential of the redox couple between the drug and the electron transfer protein, which in *Clostridia* is ferredoxin. The redox potential of such a reaction is about -450 mV. These low potentials are not found in aerobes and consequently are one factor for the selective toxicity of nitroimidazoles towards anaerobes alone. It follows that any ferredoxin-linked system of the correct potential should be capable of reducing nitroimidazoles and causing cell death. This certainly occurs even with photosynthetic systems where it has been shown that metronidazole inhibits sugar synthesis in sugar cane leaves as a consequence of inhibiting photosystem 1 (where ferredoxin is involved) but has no effect on photosystem 2, the Hill reaction.[17] Further studies in spinach chloroplasts showed a direct inhibition of ferredoxin-linked nicotinamide adenine dinucleotide phosphate (NADP) reduction and corroborative studies have shown that although metronidazole has no effect on the chemo-organotrophic growth of *Rhodopseudomonas acidophila* in the dark, it kills it when grown photo-organotrophically in the light—that is, when ferredoxin-linked systems become operative. Only in ferredoxin-operative systems, or their equivalent, are nitroimidazoles reduced.[15 18]

These events in all susceptible cells have two important consequences. The first is that reduction of nitroimidazoles creates a favourable concentration gradient in which unreduced drug enters the cell relatively rapidly as the internal concentration is decreased due to reduction. This observation, first suggested by Ings *et al*,[19] implies that entry into aerobic cells is more difficult than into anaerobes and is consequently a further factor in explaining the selective toxicity of nitroimidazoles. The second is that reduction of the nitro group is a necessary function of the drugs' cytotoxic action.

The question remains however: if reduction of the drugs via the phosphoroclastic system is not the lethal event in the cell, what is? It becomes a logical hypothesis to propose that the biochemical lesion is caused not by the original drug molecule but by a reduction product or products which act at another site. The reduction of nitroimidazoles is merely an activation mechanism therefore which only anaerobes can carry out.

Site of action

Although protein has been suggested as a possible site of action of these drugs on the basis that about 30% of labelled metronidazole binds to intracellular components, the distribution of which parallels that of protein,[20] no evidence has been published to suggest that any important protein system is inhibited. Instead, evidence has accumulated over the last few years since the suggestion of Ings *et al*[19] that DNA is the major site of action of nitroimidazoles.

Ings' initial observations showed that DNA synthesis in *T vaginalis* was inhibited as measured by incorporation of ^{14}C-labelled thymidine into perchlorate-precipitable material in both the protozoan and in *Clostridia*. This work was subsequently corroborated and extended in our laboratory and showed that DNA synthesis was not only inhibited but existing DNA was broken down in *Clostridia*, and DNA could not be extracted from drug-treated *Trichomonas* or *Clostridia*, indicating

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that nitroimidazoles cause degradation of DNA.[21] In this study it was apparent that metronidazole was a more potent degrader of DNA than tinidazole even though the latter acted sooner as a consequence of its ability to penetrate cells faster than metronidazole. Significant progress in the study of nitroimidazoles came in 1977, when it was found that chemically reduced metronidazole caused destabilisation of the DNA helix and strand breakage in vitro thus enabling model systems to be used.[22] This latter work however could be criticised on the grounds that the reducing agent itself (dithionite) could cause DNA strand breakage thus making specific measurement of the drug's action difficult.[23]

To overcome the problems of chemical reduction of nitroimidazoles we have developed an electrolytic reduction technique which enables the effect of reduced nitroimidazole on DNA to be measured directly.

Mechanism of action

Polarography of nitroimidazoles enables an assessment of the redox potentials of the drugs to be measured. These potentials can then be used to select the voltage for drug reduction ensuring that it is relevant to those potentials found in anaerobic organisms. Previous studies have already shown that if DNA is added to previously reduced drug there is no effect, indicating that the reduced intermediate responsible for DNA damage is short-lived with a half-life of less than 1 second. If electrolytic reduction is carried out in the presence of DNA at voltages not exceeding minus 1·1-1·2V, which causes DNA denaturation,[24,25] a number of techniques can be used to assess DNA damage. Generally, damage only occurs under anaerobic conditions—namely, an atmosphere of N_2—and as a consequence of the reduction of the nitro group of the drug, which can be monitored by measuring the decrease in the absorption maximum of each drug due to the nitro group.[26]

Recently, studies have been carried out on a variety of nitroimidazoles and nitropyrazoles and the damage to DNA characterised. The major techniques used in this type of study are viscometry of DNA solutions, measurements of the melting and renaturation profiles and its Tm value* using the Rowley equation,[27] measurement of the single-strand content of DNA using hydroxyapatite chromatography, measurement of the decrease in molecular weight (and thus length) by acridine-impregnated agarose gel electrophoresis, and finally the assessment of single-and double-strand breaks in DNA by sucrose gradient sedimentation ultracentrifugation.

*When DNA is heated it is converted from its normal double-stranded form (helix) to single strands (random coil) and this process is accompanied by an increase in absorbance. The midpoint of this helix-coil transition is known as the "melting" or Tm value and is expressed in degrees celsius.

Such techniques have shown that reduced drugs decrease the viscosity of DNA (which is a general index of damage), decrease the Tm, and increase the melting range (indicating a decrease in the stability of the molecule and an increase in its polydispersity) and inhibit renaturation (indicating loss of helix integrity). This loss of structural integrity of DNA may be attributed to a number of possible events, but the most obvious is that of scission of the phosphodiester backbone—that is, strand breakage. Confirmatory evidence for strand breakage comes from studies which show a drug-induced increased single-strand content of DNA, its decreased molecular length and weight, and direct measurement of single- and double-strand breaks by sucrose gradient sedimentation.[27-30] These effects have been observed with metronidazole, misonidazole, 4-nitroimidazole, M & B 4998 and 8609 RP (for structures see figure). The overall mechanism of action of all these drugs therefore is to decrease the stability and integrity of DNA by strand breakage.

Metronidazole and misonidazole are presently being evaluated as radio-sensitisers of hypoxic tumours,[31] and the process of radiosensitisation has been extensively studied. This process has many features in common with the cytotoxic properties of nitroimidazoles, including the parameters of nitro-group reduction and DNA strand breakage. Where the processes appear to differ is in the speed and temperature dependence. Thus radiosensitisation is a fast process (about 10-30 milliseconds) as is radiation damage (10 milliseconds) compared with radioprotection mechanisms (850 milliseconds) and cytotoxic effects (about 1 second) and is temperature-independent, whereas cytotoxicity is relatively slow and temperature-dependent. It is now well established that the damaging agent of nitroimidazoles as radiosensitisers is the radical anion ($-NO_2^{\bar{\cdot}}$) since the efficiency of radiosensitisation of nitroimidazoles can be correlated with their one-electron redox potentials.[32,33]

However, if the efficiency of strand-breakage of DNA in an electrolytic reduction in-vitro system is compared with the one-electron redox potential or the polarographic half-wave potential no obvious correlation exists[27] indicating that the cytotoxic effect towards anaerobes is not due to the nitro radical anion. Recent observations in our laboratory[30] show that reduced nitroimidazole-induced damage of DNA can be prevented by certain aminothiols, notably cysteamine and cystamine, and that cysteamine acts as a radical scavenger whereas cystamine does not but behaves as a radioprotector.

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This leads to the conclusion that the reduced agent responsible for DNA damage is at least further reduced beyond the radical anion stage. Precisely what this agent is has not been established yet because of its (their) inherent instability and short half-life. However, the implications of this cytotoxic agent are important for the anticancer action of the drugs since it has been established that the killing effect of the drugs in conjunction with radiation is greater than can be accounted for by radiosensitisation alone. The evidence for this comes from studies in which both normal (oxic) and hypoxic tumour cells are killed.[34] Since the radiosensitisation effect occurs only in hypoxic cells the killing of oxic cells can only be accounted for by a cytotoxic mechanism which does not involve free radicals.

The model electrolytic reduction system is applicable therefore for the study of both the mechanisms of cytotoxicity in anaerobes and in hypoxic cells.

Molecular basis of selective toxicity

Studies of the relative damage done by a number of nitroimidazoles results in two important characteristics of drug action. The first is that the amount of damage varies with the base composition of DNA and the second is that the amount of protection afforded by cysteamine depends on the redox potential of the drug. This latter point is important since the efficiency of action on DNA depends on the degree of strand breakage and aminothiol protection within the cell.

As regards the base composition of DNAs, it can be shown that damage is directly proportional to the A + T content irrespective of what technique is used to measure DNA damage (Edwards *et al,* unpublished observation) and that, using a variety of chromatographic techniques, it appears that thymidine phosphates are specifically released from DNA (Knox *et al,* unpublished observations). These results also argue against a free radical being responsible for such selective DNA damage. Precisely how specific thymidine phosphate release is achieved is not known and will require the elucidation of the active intermediate before this process is understood, but it does enable a molecular basis of selective toxicity to be formulated. Thus, one would expect nitroimidazoles to be particularly selectively toxic to anaerobic organisms which have A + T-rich DNAs and somewhat less so in G + C-rich organisms. The protozoans, *T vaginalis* and *Entamoeba histolytica,* have A + T contents of 71% and 62-78% respectively[35-38] and *Bacteroides* have A + T contents of about 59-61%. These organisms are the main causes of disease for which nitroimidazole drugs have major clinical application. In contrast, *Rhodospirillum* and *R acidophila* have relatively low A + T contents (38% and 33% respectively) and these organisms are about 25 times less susceptible to nitroimidazoles than the protozoa or bacteria based on relative MICs. The implications of this will be discussed in detail elsewhere, but it is obvious that the particular effectiveness of such A + T-specific drugs against A + T-rich DNA is highly germane to their spectrum of activity or taxonomic specificity.[15]

Resistance and drug inactivation

There have been many reports of trichomonads, including *T vaginalis,* which are resistant to metronidazole or other nitroimidazoles or both.[39-41] Most of these reports are either of no clinical significance, because resistance levels are well below therapeutically obtainable concentrations, or the result of non-anaerobic conditions.[40 41] Because the presence of O_2 in cultures raises the redox potential to positive values drug reduction does not occur and MIC values will increase, erroneously indicating the presence of resistant organisms. The underlying biochemical mechanisms for this may involve carboxylation reactions in *T vaginalis.*[42] However, truly resistant organisms have been developed in the laboratory and these are cross-resistant to a wide variety of nitroimidazoles.[39]

It is unusual that no resistant organisms of clinical significance have arisen during 20 years' use with either metronidazole or any other nitroimidazoles. This would suggest that a single-gene change conferring resistance is itself lethal and indicative of a mechanism and site of action where gene mutation is not compatible with cell survival. However this does not necessarily mean that resistance to nitroimidazoles is impossible; indeed several reports indicate that resistance to nitroimidazoles is possible but not to an extent that would have any clinical significance. The major, albeit rare, occurrence is that of drug inactivation which manifests itself during treatment. In the case of *T vaginalis,* the organism from refractory patients is invariably found to be sensitive in vitro. The reason for this is the presence of other organisms in the vagina, notably *Streptococcus faecalis, Escherichia coli, Klebsiella,* and *Mimae,* some of which can alone, or in combination, absorb sufficient amounts of drug to lower its effective concentration in the environment below that required for therapeutic purposes. Recent studies have shown that normally aerobic organisms are capable of absorbing amounts of metronidazole without their viability being significantly affected

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both in aerobic and anaerobic environments.[43 44] This inactivation is a feature both of 5- and 2-nitroimidazoles and may well be relevant to inactivation in other clinically relevant situations, for example, anaerobes in pus.

Clearly, because of the molecular basis of action of nitroimidazoles, resistance could not occur by way of an altered DNA or enzymes involved in DNA biosynthesis. Theoretically however there are mechanisms by which resistance to nitroimidazoles could be accomplished. Since entry into the cell is a prerequisite for drug activation (by reduction) an alteration in membrane permeability to exclude nitroimidazoles could lead to resistance. Attractive though this mechanism may be it must be remembered that nitroimidazoles are small molecules which enter the cell passively and there is no competition for uptake by other imidazoles, for example, histidine, histamine (Edwards, unpublished observations). Any alteration in membrane permeability to exclude nitroimidazoles could well also prevent uptake of histamine or histidine and other small molecules and be lethal to the cell.

A more logical mechanism of resistance would be based not on entry to the susceptible cell but on non-activation of the drug, that is, no reduction of the nitro group. Since the mechanism for reduction lies in the pyruvate phosphoroclastic reaction one would expect a mutation in one of the enzymes of this complex such that the nitroimidazole is not accepted as a substrate. It must be pointed out that resistance to nitrofurans occurs by way of an altered nitroreductase enzyme and that these drugs are not reduced via a ferredoxin-linked mechanism because their redox potential is too positive.[45 46]

It is of great interest that Britz and Wilkinson[47] have recently produced metronidazole resistance in *Bacteroides* using powerful mutagens, but the treated organisms have very much reduced levels of pyruvate dehydrogenase, a key enzyme involved in the phosphoroclastic reaction. Although no resistance mechanisms of this type in protozoa have been reported there have been reports of metronidazole-resistant *Bacteroides* resulting from long-term therapy,[48] and it would be interesting to discover the precise mechanism of resistance of those organisms which are resistant to clinically significant concentrations of drug.

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